A COMPARATIVE STUDY OF SV40-TRANSFORMED FIBROBLAST PLASMA MEMBRANE PROTEINS LABELLED BY ENZYMATIC IODINATION OR WITH TRINITROBENZENE SULFONATE

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1. Introduction

Information concerning the structure of plasma membranes is of critical importance in the understanding of molecular mechanisms controlling many leading functions in eukaryotic cells, particularly cell–cell interactions and growth control. Selective labelling has recently joined conventional methods as a means of analysing components exposed on the cell surface under physiological conditions [1–5]. In addition to providing information relating to the location of a particular molecule within the membrane architecture, it permits a distinction to be made between true surface components and those adsorbed as a result of the handling involved during cell fractionation. Iodination catalyzed by lactoperoxidase [4,5] and sialylation by surface sialyl-tranferases [6] are the two enzyme labelling methods that appear to give the best results in this connection. They have recently been flanked by a method for selective labelling of amino groups exposed on the membrane surface using trinitrophenyl groups. This method has the advantage of permitting subsequent single step purification of the labelled membrane proteins with a reverted immunoadsorbent consisting of insoluble specific antibodies, and has been reported for red cell membrane labelling [7]. The present paper describes its use in the study of SV-40/3T3 fibroblast membrane proteins. Comparison is made with proteins solubilized from isolated plasma membranes and with surface proteins labelled by enzymatic iodination.

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2. Material and methods

2.1. *Cells*

SV-40 transformed mouse 3T3 fibroblasts [8,9], kindly supplied by Dr G. Todaro, were grown in Eagle's MEM (Difco) in large (200 cm^2) glass bottles. At densities close to 3×10^4 cells/cm^2, cells were carefully washed with serum-free medium and detached from the glass by very gentle scraping with a rubber tipped spatula. Cell viability was determined by the trypan blue exclusion test [10] before and after labellings, and only preparations containing more than 90% of living cells were used for the experiments. Internal labelling of membrane proteins was performed by incubation of cells in medium supplemented with ^{14}C-L-amino acids (1 mCi/l) for 24 hr.

2.2. *Isolation of plasma membranes*

For the isolation of plasma membranes the zinc method of Warren et al. [11] as modified by Brunette and Till [12] and Sheinin and Onodera, was employed. This method has succesfully been used to study 3T3 and SV-40/3T3 plasma membranes [13,30]. Briefly, saline washed cells were incubated in $ZnCl_2$ 10^{-3} M for 15 min at room temperature and for 5 min in ice. Cells were than disrupted by Dounce homogeneization with repeated phase-contrast microscope controls. Whole cell ghosts and plasma membranes debris were then separated from subcellular organelles by a two-phase dextranpolyethylenglycol system, sodium phosphate buffered at pH 6.5. ^{125}I or trinitrophenyl-labelled plasma membranes were processed as above.

2.3. *Electron Microscopy*

The procedures used for fixing, embedding and staining membrane pellets are described in detail in ref. [14]. Observations were performed in a Philips EM-300.

2.4. *Enzymatic Iodination*

Washed cells (2.5×10^6/ml) were suspended in 10 ml of phosphate buffered saline pH 7.2 and 1 mg of lactoperoxidase, and 1 mCi of carrier-free radioactive sodium iodide (^{125}I) was added. The suspension was incubated with gentle stirring at 30°C for 10 min; during this time 85 μM (total) of hydrogen peroxide were added dropwise, according to the method of Marchalonis et al. [15]. The hydrogen peroxide was added extremely slowly and in such a small amount in order to minimize both oxidation of surface components and cell penetration of reactive iodine. After 10 min the reaction was stopped by 10^{-3} M sodium azide and by chilling at 0°C; labelled cells were washed twice with phosphate buffered saline and subjected to cell fractionation for isolation of membranes.

2.5. *Trinitrobenzene sulfonate labelling and purification of surface proteins*

Selective surface labelling of SV-40 3T3 fibroblast was performed by the dialysis membrane bag technique previously described [7], with minor modifications. Briefly, washed cells were suspended in Earle's solution and placed in a dialysis bag dipped in a 15 mM solution of trinitrobenzene sodium sulfonate, buffered at pH 7.3 in Earle's solution. Both the cell suspension and the trinitrobenzene sulfonate solution were heated at 37°C and stirred for 30 min. The reaction was stopped by iced 0.75 M glycine in Earle's solution and cells were washed with the same solution until colorless supernatants were obtained. After cell fractionation, labelled membrane proteins were solubilized by overnight incubation at 4°C in 10 vol of 0.1 M barbital buffer pH 8.6 containing 1% sodium deoxycholate, followed by ultracentrifugation at 100 000 *g* for 1 hr. After extensive dialysis against 0.05 M barbital buffer pH 8.2, 0.1% deoxycholate, trinitrophenyl-labelled proteins were purified by affinity chromatography, using purified rabbit anti-dinitrophenyl antibodies covalently linked to Sepharose 4B by the cyanogen bromide technique [16–17]. The reasons for using anti-dinitrophenyl and not anti-trinitrophenyl antibodies have been discussed in a previous paper [7]. Each immunoadsorbent column was washed immediately before use with 1 M acetic acid and equilibrated with 0.05 M barbital buffer pH 8.2, 0.1% deoxycholate. Solubilized membrane samples were then slowly passed through the columns, followed by extensive washings with buffer. All these steps were performed at 4°C in order to prevent proteolytic degradation. Trinitrophenyl-labelled molecules were eluted with 8 M urea–1 M acetic acid, dialyzed against water, lyophilized and dissolved in sodium dodecyl sulfate for disc-gel electrophoresis.

2.6. *Acrylamide gel electrophoresis*

The sodium dodecyl sulfate disc-gel electrophoresis method of Maizel [18] was followed. Isolated membrane samples or purified trinitrophenylated molecules, containing 80–100 g of proteins, were electrophoresed in 9% acrylamide gels, with a 3% upper spacer. Gels were fixed, Coomassie Blue stained and scanned in a Joice-Loebl 'Chromoscan' densitometer. Calibration of the system was achieved by simultaneous separate runs of the following standards: cytochrome *c* (mol. wt. 12 000), concanavalin-A (mol. wt. 27 000), ovoalbumin (mol. wt. 43 000), bovine serum albumin (mol. wt. 67 000) and lactoperoxidase (mol. wt. 78 000) [19–21]. ^{125}I-labelled proteins were determined by slicing the gels and counting each slice in a Nuclear-Chicago γ-counter.

3. Results

The electron microscope examination of isolated SV-40 3T3 plasma membranes obtained by the zinc method revealed a uniform appearance of large membrane fragments, among which a few other subcellular organelles (mainly small vescicles and ribosomes) were occasionally observed. Each plasma membrane section was coated on one side with a relatively thick layer of filamentous material (fig. 1), which is very often reported to contaminate membrane fractions obtained by the most commonly used methods. Judging by morphological criteria, our membrane preparations showed the standard levels of purity obtained by this or similar methods of fractionation.

Sodium dodecyl sulfate–mercaptoethanol solubilized

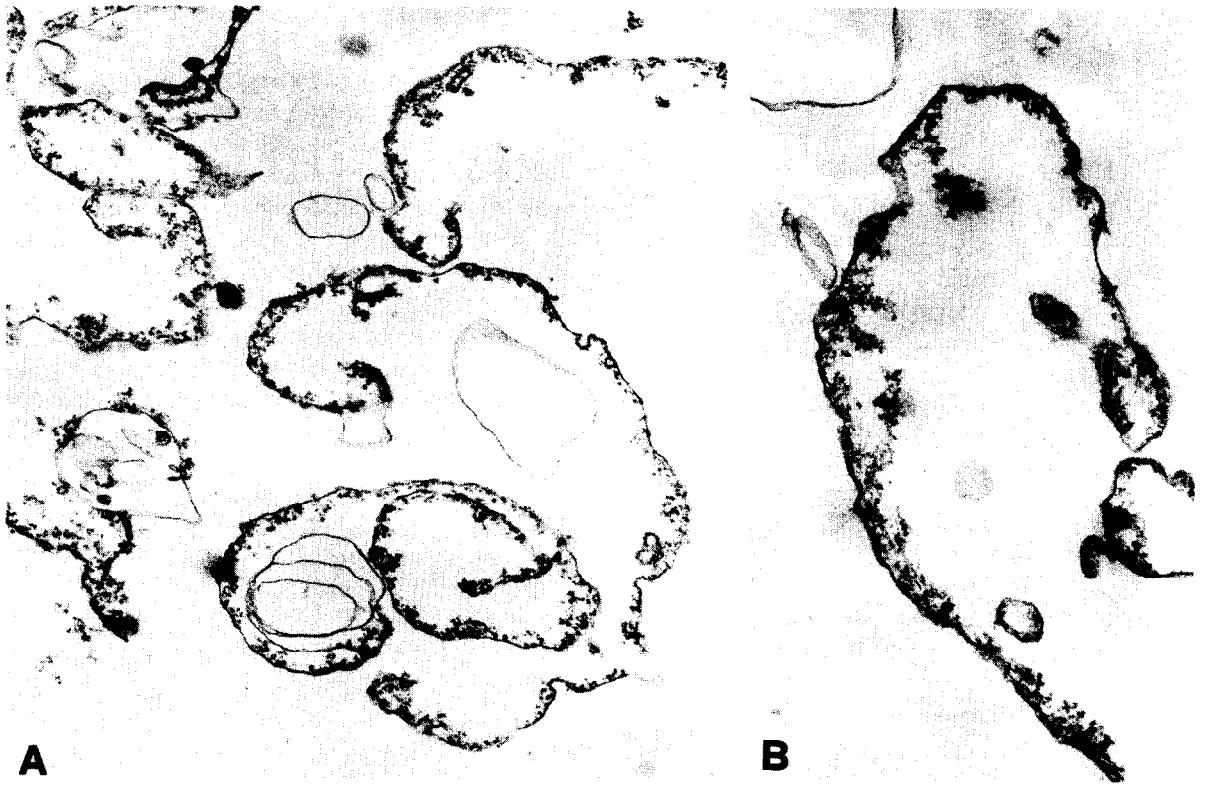
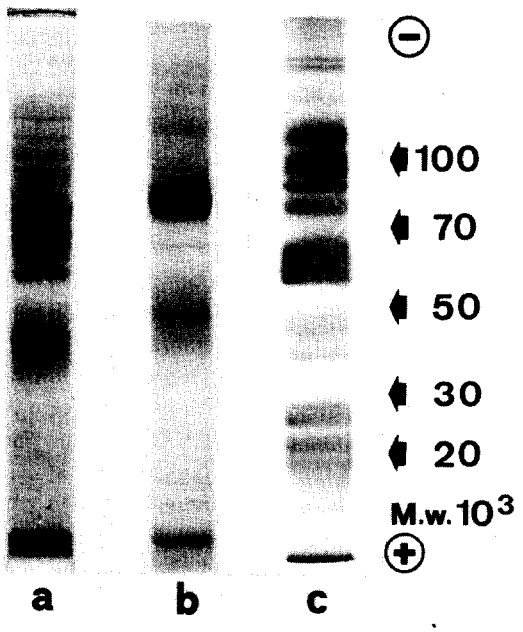


Fig. 1. Electron micrograph of SV-40 transformed 3T3 plasma membranes isolated by a two-phase dextran–polyethylen glycol system. Thin section stained with Mg-uranyl acetate and lead citrate. (A: 54 000 X, B: 108 000 X.)



membrane pellets electrophoresed on 9% acrylamide gels resolved in a high number of bands of mol. wt. between 220 000 and 10 000, which gave rise to a very complex pattern (figs. 2 and 3).

SV-40 3T3 surface proteins labelled by enzymatic iodination as described under methods and separated in disc-gel electrophoresis gave a much simpler pattern

Fig. 2. Coomassie blue stained polyacrylamide gels of SV-40/3T3 plasma membrane proteins. A) Whole plasma membranes dissolved by sodium dodecyl sulfate–β-mercaptoethanol. B) Surface membrane proteins labelled with trinitrobenzene sulfonate and purified by affinity chromatography on anti-dinitrophenyl antibodies–Sepharose. C) Plasma membrane proteins labelled by trinitrobenzene sulfonate after solubilization by 1% sodium deoxycholate. After being labelled, trintrophenylated proteins were purified by immunoadsorption as above. The densitometric profiles of each gel are given in figs. 3 and 4.

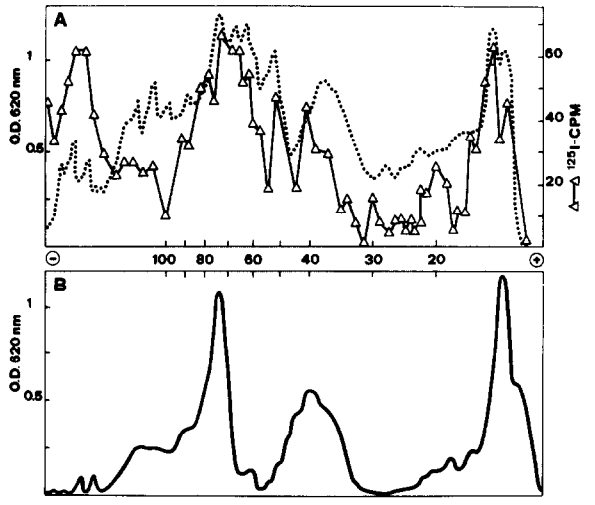


Fig. 3. Electrophoretic profiles of plasma membrane proteins labelled by enzymatic iodination or trinitrobenzene sulfonate. A) The ^{125}I-labelled protein pattern (▲——▲) is superimposed to the densitometric profile obtained from the stained gel (. . . . .). Twenty–twenty-five incompletely resolved peaks of radioactivity were seen spread over the whole length of the gel. B) Cell surface proteins selectively labelled by trinitrobenzene sulfonate and purified by reverted immunoadsorption. The molecular weight of the three mayor peaks is included between 90–70 000, 50–35 000 and 16–17 000. All bands were internally labelled by ^{14}C-amino acids and were identifiable with corresponding bands present in the pattern obtained by enzymatic iodination.

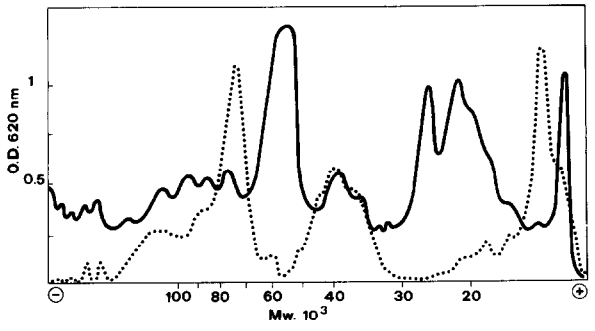


Fig. 4. Electrophoretic pattern of purified trinitrophenylated plasma membrane proteins which were solubilized by deoxycholate prior to trinitrobenzene sulfonate labelling (——). The dotted line shows the profile of cell surface proteins selectively labelled with the reagent in physiological conditions. The two gels were separately run and the densitometric patterns superimposed.

than that obtained with whole plasma membrane preparations. At least 20–25 incompletely separated peaks of radioactivity were seen, subdivided into seven major groups of components (fig. 4). 33% of the total radioactivity was found to be linked with components of mol. wt. between 90 000 and 50 000, while the other two major groups, which accounted for 20% and 15% of the total radioactivity, had a mol. wt. between 180 000 and 160 000, and 16 000 and 12 000 respectively.

SV-40 3T3 surface molecules were then labelled by trinitrobenzene sulfonate at 37°C for 30 min as previously described [7] and purified from unlabelled membrane components on a reverted immunoadsorbent.

The purified trinitrophenyl-proteins, subjected to acrylamide electrophoresis, and stained with Coomassie Blue, resolved into a discrete number of bands, grouped in three major peaks of mol. wt. between 90 000 and 70 000, 50 000 and 35 000 and 16 000 and 12 000. All bands found in purified trinitrophenyl-labelled preparations contained ^{3}H-amino acids and were identifiable with corresponding bands given by whole plasma membranes. Moreover, all three groups had a corresponding peak of radioactivity in the ^{125}I-labelled patterns (fig. 3). To demonstrate that trinitrobenzenesulfonate did not label internal cell components and that trinitrophenylgroups were selectively linked to exposed surface plasma membrane molecules, trinitrophenyl-labelled cells were disrupted by simple homogeneization and centrifuged at 100 000 *g* for 1 hr. The supernatant representing the soluble cytoplasmic fraction was passed through the immunoadsorbent. In this case, the urea–acetic acid elution did not yeld a significant amount of material, showing that cytoplasm proteins were not labelled in a detectable way, and that no component was unspecifically retained by the column and released during elution. Similar negative results were obtained when a preparation of native membrane proteins (i.e. not treated by any labelling reagent) was chromatographed through the anti-DNP immunoadsorbent. By using ^{14}C-amino acid internally labelled membrane preparations, it was found that the amount of TNP-labelled proteins eluted from the immunoadsorbent by urea–acetic acid varied between 8–10% of the total amount of radioactivity solubilized from membranes by deoxycholate. Less than 20% of the bound

radioactivity was retained by the immunoadsorbent after elution. Since radioactive trinitrobenzene sulfonate is not readly available, it has not been possible to determine how much TNP-labelled protein was not retained by the affinity column in this system. However control experiments performed with TNP_2-ovoalbumin and TNP_{15}-bovine serum albumin showed that in both cases the anti DNP-column retained as much as 80–85% of the TNP-protein applied.

The unlikely possibility that trinitrobenzenesulfonate reacted only with a restricted class of proteins was ruled out by labelling a cell homogenate with the dialysis bag system, and by further processing the labelled material with the immunoadsorbent, after chromatography on Sephadex G-25 to remove the unreacted trinitrobenzene sulfonate molecules. In this case, the purified trinitrophenyl-labelled proteins gave an extremely complex electrophoretic pattern, similar to that given by the whole homogenate. A similar possibility at the level of membrane proteins was excluded by treating SV-40 3T3 cells with trinitrobenzene sulfonate in conditions producing specific surface labelling. Labelled plasma membranes were then solubilized and chromatographed on the immunoadsorbent as usual. The first peak effluent from the column (i.e. the unlabelled membrane proteins) was then labelled with trinitrobenzene sulfonate under standard conditions, chromatographed on a second anti-dinitrophenyl immunoadsorbent and eluted with urea-acetic acid. The electrophoretic pattern obtained, (fig. 1 and 4), showed that after deoxycholate solubilization at least 20 more components were labelled by trinitrobenzene sulfonate.

4. Discussion

Electrophoresis of proteins solubilized from isolated membranes of SV-40 transformed 3T3 fibroblasts and of those labelled by enzymatic iodination or trinitrobenzene sulfonate gives different results. In each group, the gel patterns were highly reproducible, as can be seen by the results obtained from three different sets of experiments.

The pattern for whole membranes purified in accordance with one of the more commonly used methods is very complicated. Its heterogeneity is comparable with that described for various types of membrane isolated with this or other methods [23–27] The morphological analysis of isolated membranes always showed that they had some filamentous material attached on the cytoplasm side, and thus some of the electrophoretic bands seen may be attributed to this material. Labelling with enzymatic iodination gives a simpler pattern with 20–25 different protein peaks distributed along the entire gel length, indicating an extensive molecular weight range.

Surface labelling with trinitrobenzene sulfonate followed by purification gives an even simpler pattern, with proteins in three main mol. wt. groups: 90–70 000, 50–35 000 and 16–12 000. It is clear, therefore, that proteins of intermediate weight labelled by enzymatic iodination were missed with this method.

Several advantages are offered by selective surface labelling with trinitrobenzene sulfonate, some of which are not obtained with iodination. Both methods ensure that a distinction can be made between molecules exposed on one or both plasma membrane surfaces and those masked in the membrane core and hence unreactable by the reagent. Moreover, both methods offer a good degree of discrimination between true membrane components and molecules adsorbed aspecifically on the membranes during preparative handlings. Discrimination may be blunted by the fact that membranes may form tight bonds with some serum proteins of the medium. However artifacts of this kind can be avoided in a number of simple ways [28].

The main advantage peculiar to trinitrobenzene sulfonate labelling is that the method is preparative as well as analytic. Laborious conventional procedures of plasma membrane purification are eliminated by using an immunoadsorbent to isolate trinitrophenylated membrane molecules from all other non-labelled cell components.

Comparison with enzymatic iodination showed that ^{125}I bonds with a greater number and a more heterogeneous class of proteins: 20–25 electrophoretic peaks as opposed to the 7–9 observed with trinitrobenzene sulfonate labelling. Iodine–protein bonds are formed with the imine groups of histidine or substitution of the phenol hydroxyl of thyrosine residues [5], whereas trinitrobenzene sulfonate bonds by means of nucleophilic substitution. This is primarily directed to the ϵ-amino groups of lysines which, in most proteins, are certainly as numerous as the tyrosine

or histidine residues. Two explanations can be offered for the fact that trinitrobenzene sulfonate labelled fewer membrane proteins. In the first place, the size and charge of trinitrobenzenesulfonate prevent it from penetrating the outer surface molecular layer of plasma membranes as deeply as free iodine. Moreover, at neutral pH, TNB-sulfonate binds protein amino groups much slower than iodine tyrosyl-residues; in our experimental conditions, in fact, saturation of the cell surface was not reached. The purified TNP-pattern may, of course, be artificially simplified by selective action on the part of the immunoadsorbent, since this may bind or release proteins labelled with a different number of groups in a different manner. This possibility, however, would seen to be ruled out by the experiments with TNP_2-ovoalbumin and TNP_{15}-bovine serum albumin, which were bound by and eluted from the immunoadsorbent to the same extent. In any event, is clear that the simplicity of the trinitrobenzene sulfonate labelling pattern, whether artificial or not, makes this method extremely useful for the study of plasma membranes.

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References

[1] Maddy, A. H. (1964) Biochim. Biophys. Acta 88, 390–399.
[2] Berg, H. (1969) Biochim. biophys. Acta 183, 65–78.
[3] Bretscher, M. (1971) J. Mol. Biol. 58, 755–781.
[4] Philips, D. and Morrison, M. (1971) Biochemistry 10, 1766–1771.
[5] Hubbard, A. and Cohn, Z. (1972) J. Cell Biol. 55, 390–405.
[6] Comoglio, P. M. and Warren L. manuscript in preparation.
[7] Tarone, G., Prat, M. and Comoglio, P. M. (1973) Biochim. Biophys. Acta 311, 214–221.
[8] Todaro, G. J. and Green, H. (1963) J. Cell Biol. 17, 299–313.
[9] Todaro, G. J. and Gree, H. (1964) Virology 23, 117–119.
[10] Takahashi, T., Old, L. and Boyse, E. (1970) J. Exp. Med. 131, 1325–1332.
[11] Warren, L., Glick, M. C. and Nass, M. (1966) J. Cell Physiol. 68, 269–287.
[12] Brunette, D. M. and Till, L. E. (1971) J. Membr. Biol. 5, 215–224.
[13] Sheinin, R. and Onodera, K. (1971) in: The biology of oncogeneic viruses (Silvestri, L. ed) pp. 274–285, North Holland.
[14] Meldolesi, J., Jamieson, J. D. and Palade, G. E. (1971) J. Cell Biol. 49, 109–129.
[15] Marchalonis, J. J., Cone, R. E., Sauter, V. (1971) Biochem. J. 124, 921–927.
[16] Givol, D., Weinstein, Y., Gorecki, M. and Wilchek, M. (1970) Biochim. biophys. Res. Comm. 38, 825–830.
[17] Axen, R., Porath, J. and Ernback, S. (1967) Nature, 214, 1302–1304.
[18] Maizel, J. V. (1971) in: Methods in Virology, V (Maramorosch, K. and Koprowski, H., ed) p. 315, Academic Press, New York.
[19] Hardman, K. D., Ainsworth, C. F. (1972) Nature N.B. 237, 54–55.
[20] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[21] Morrison, M., Bayse, G. and Danner, J. G. (1970) in: Biochemistry of the phagocytic process (Schultz, J., ed) p. 5166, North Holland.
[22] Tischler, P. V. and Epstein, C. J. (1968) Anal. Biochem. 22, 89–98.
[23] Scher, I. and Barland, P. (1972) Biochim. Biophys. Acta, 255, 580–588.
[24] Trayer, H. R., Nozaki, Y. A. and Tanford, C. (1971) J. Biol. Chem. 246, 4485–4496.
[25] Fairbaks, G., Steck, T. C. and Wallach, D. F. (1971) Biochemistry 10, 2605–2620.
[26] Neville, D. M. and Glossmann, H. (1971) J. Biol. Chem. 246, 6335–6349.
[27] Greenberg, C. S. and Glick, M. C. (1972) Biochemistry 11, 3680–3685.
[28] Chiarugi, U. P. and Urbino, P. (1973) Biochim. biophys. Acta. 298, 195–205.
[29] Sheinin, R. and Onodera, K. (1972) Biochim. biophys. Acta, 274, 49–63.